DTT Inhibition of Vitamin C uptake decreases Collagen II expression

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Introduction:

Stem cells differentiate into tissues via various inter- and intra-cellular signals. Collagen II has been shown to correlate with an induction of differentiation from Mesenchymal Stem Cells (MSCs) to mature Chondrocytes[1]. Moreover, Vitamin C has been shown as a cofactor in collagen synthesis, meaning that without Vitamin C[2], collagen II (an indicator of the presence of mature chondrocytes) ceases to form. Previous studies have also indicated that the addition of Ascorbate (vitamin C) acts as an inhibitor of Chondrocyte dedifferentiation[3]. In this experiment we thought it would be interesting if we added Dithiothreitol (DTT), a compound that changes the conformation of Vitamin C, such that it cannot be processed by the cell[4]. We predict that since vitamin C is a cofactor in collagen polymerization, that adding DTT would decrease the expression levels of collagen II because this necessary cofactor is not present, inducing cell de-differentation because this signal is no longer present. RT PCR results show that indeed, in the presence of DTT collagen II levels exhibited a two-fold decrease, confirming that Vitamin C is a necessary component of collagen synthesis.

Methods:

We grew cells as concentration of $2.01*10^6$ cells/mL, used the standard Sigma Aldrich 1% alginate beads, and added DTT to one of our solutions at a concentration of 0.1 mM. Our cultures of alginate beads were very fragile, for only 6 beads in the –DTT cultures and 12 beads in the +DTT cultures by the time we isolated our protein and mRNA. Prior to putting samples into the RT-PCR reaction, we measured a total mRNA concentration using A_{260} and A_{280} spectroscopy. The A_{260} levels of both samples were .006 after a 100 fold dilution, and the A_{280} levels were around 0 for both samples. Calculations reveals a final concentration of 24 ng/uL and therefore, in order to add the standard 100ng, 4.2 uL of crude lysate was used.

Results:

RT PCR results show decrease in levels of Collage II expression: Cell viability assays revealed that most of the cells were alive in the alginate beads at the time of RNA isolation (Data not shown). After isolation of the mRNA and RT PCR, the brightness of the bands was measured ImageJ program. As shown in Figure 1, from the RT-PCR results, there is a significant decrease in the expression of Collagen II, indicated by a dimming of the upper band in the lane with the +DTT sample. The ImageJ program produced, after statistical normalization by background subtraction, CNI/GADPH ratios of 1.96 and 2.38 in the –DTT and +DTT samples, respectively and CNII/GADPH ratios of 1.13 in –DTT and 0.512 in +DTT samples.

ELISA analysis reveals very little expression of collagen: Plotting the absorbance of known concentration of collagen I/II, yielded a standard lines, which we used to determine the

concentration of our samples. As shown in Figure 2, the concentration of our experimental samples in both the –DTT and +DTT were extremely low (negative concentrations), producing no meaningful comparison.

Discussion:

Our RT PCR results indicated that there was more than a twofold decrease in the CNII/GAPDH ratio upon the addition of DTT, which prevents Vitamin C uptake in the cell. This result is indicative of less expression of Collagen II, which probably means that cells have de-differentiated as Collagen II expression correlates with a Chondrocytic phenotype. This result verifies previous studies which have shown that decreasing vitamin C levels induces Chondrocyte de-differentiation, for adding DTT is very similar to taking away vitamin C. Our ELISA results were unfortunate in that they did not give us any meaningful concentration because there were too few cells, but perhaps if we repeat the experiment with more cells then we can assay the relative levels of expression of Collagen I/II in our cells. Future experimentation might include treating Mesenchymal Stem Cells with DTT and seeing whether this would produce is similar phenotype of non-differentiation. Another experiment that might prove useful is if we treated samples with multiple different concentrations of DTT. If we obtained a gradient of values for CollagenII expression levels, then we could definitively prove that our results were correct. Lastly, we could expose cells that have mutations of the enzyme responsible for collagen synthesis and see whether adding DTT would have any result on this strain of cells. Whatever the case, understanding the biochemical mechanism that is responsible for differentiation of Stem cells into chondrocytes provides the foundation for biological synthesis of cartilage.

References:

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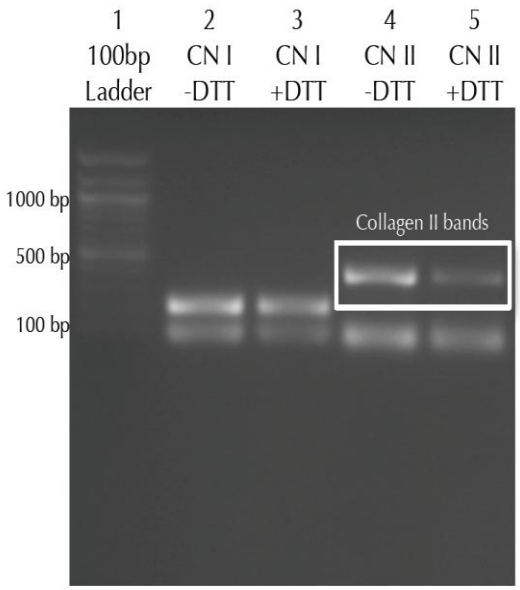

Figure 1: **RT-PCR gel electrophoresis indicates decreased expression of Collagen II upon addition of DTT.** After RT-PCR reactions were run with the crude lysate samples of mRNA normalized for concentration, band brightness was analyzed in the Excel. Statistical analysis reveals a brightness, after background subtraction, of 74.172 and 37.654 for the CNI and GAPDH in the –DTT sample and 55.542 and 23.225 for the CNI and GADPH in the +DTT sample. For the Collagen II analysis, a brightness of **64.558** (CNII) and 56.738 (GAPDH) was observed in the –DTT sample and **20.360** and 39.705 were observed in the +DTT sample. These values correspond to our expected outcomes, for the upper band is much dimmer in the +DTT/CN II lane.

Figure 2: **ELISA analysis reveals a low concentration of both Collagens I and II**. Absorbance values were gathered and background was subtracted in Excel to obtain normalized absorbance values. Known concentrations were plotted with their absorbance values and a standard curve was generated with R^2 = .9788 for Collagen I and R^2 = .9639 for Collagen II. The absorbance of the experimental samples was analyzed using these standard curves to obtain values for their concentration. As shown on the graph, the experimental samples (◆) all had values that were less than or very close to 0, indicating that there was no significant amount of Collagens I nor II in any of our cultures.